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(54) Title: CARDIOVASCULAR DISEASE RISK ASSESSMENT

(57) Abstract

Improved cardiovascular risk assessment is achieved by determining the level of asymmetric N,N–dimethyl arginine ("ADMA") in the physiological fluid of a subject. Increased levels of ADMA over normal indicate a risk of cardiovascular disease. Binding proteins are provided which have a high affinity for ADMA, which have a substantially reduced affinity for congeners encountered in physiological fluids, so as to reduce the incidence of false results. The results observed with ADMA may be buttressed with the determination of other indicators, conveniently providing a kit which includes the reagents for the determination of ADMA and such other reagents.

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# CARDIOVASCULAR DISEASE RISK ASSESSMENT

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of Provisional Application serial no. 60,045,278, filed May 1, 1997.

## INTRODUCTION

### BACKGROUND

1. EDNO regulates vascular tone.

EDNO is the most potent endogenous vasodilator known, and, by its effect upon vascular resistance and cardiac contractility, is a major regulator of blood pressure (Moncada and Higgs, 1993; Cooke and Dzau, 1997). NO exerts its effects as a vasodilator, in part, by stimulating soluble guanylate cyclase to produce cGMP. A deficiency of EDNO (as in the endothelial NOS knockout, or with administration of NOS antagonists), causes hypertension (Dananberg et al., 1993; Shesely et al., 1996). An overproduction of NO (as in sepsis), causes hypotension and cardiovascular collapse (Rees et al.,1990; Petros et al., 1991).

NO is released from the endothelium in response to a wide variety of physiologic stimuli. For over a century physiologists have recognized that as blood flow increases through a conduit vessel, the vessel dilates. This flow-mediated vasodilation is dependent upon the integrity of the endothelium, and is largely due to

the release of EDNO in response to endothelial shear stress (Cooke et al., 1990; Cooke et al., 1991a). Endothelial cells also respond to pharmacological stimuli. Most vasoconstrictors, such as norepinepherine, 5-hydroxytryptamine, and angiotensin II, also stimulate NO release by the endothelium (Moncada and Higgs, 1993; Cooke and Dzau, 1997).

In this way the endothelium modulates vascular contractility. These responses have physiological consequences. For example, during exercise or with mental stress, myocardial oxygen demands increase. In normal individuals the epicardial coronary arteries dilate to accommodate the need for increased coronary blood flow. By contrast, individuals with coronary artery disease have a dysfunctional endothelium with reduced EDNO production and/or activity. In these individuals, a paradoxical coronary artery constriction is observed with exercise or mental stress that contributes to reduced coronary blood flow, resulting in myocardial ischemia (Cox et al., 1989; Zeiher et al., 1989).

In addition to its role as a vasodilator, EDNO is potent inhibitor of vascular smooth muscle (VSM) proliferation. The proliferation of cultured VSM cells is inhibited by exogenous NO donors and cGMP analogues (Garg and Hassid, 1989). Gene transfer of endothelial NOS into the balloon-injured rat carotid artery in vivo demonstrably increases NO release for days after the transfection, and significantly reduces myointimal hyperplasia due to proliferation of intimal vascular smooth muscle cells (von der Leyen, et al., 1995).

EDNO also affects vascular structure by inhibiting the interaction of circulating blood elements with the vessel wall. Platelet adherence and aggregation is inhibited by EDNO (Radomski et al., 1987; Stamler et al., 1989). The adherence and infiltration of leukocytes into the vessel wall during experimental inflammation is reduced by exogenous administration of NO donors, and is enhanced by administration of NOS antagonists (Lefer et al., 1993; Gaboury et al, 1993).

To summarize, in states of vascular injury or inflammation, a deficiency of NO contributes to thrombosis, leukocyte infiltration, and vascular smooth muscle proliferation.

2. The role of NO in atherosclerosis

Atherosclerosis is the major cause of disability in this country and is responsible for 500,000 deaths annually due to coronary artery disease and cerebral vascular attack. Atherosclerosis is accelerated by hyper-cholesterolemia, hypertension, diabetes mellitus, tobacco use, elevated levels of lipoprotein(a) ("Lp(a)") and homocysteine. Intriguingly, all of these disorders are characterized in humans by an endothelial vasodilatory dysfunction well before there is any clinical evidence of atherosclerosis (Cooke and Dzau, 1997). In all of these conditions, the abnormality appears to be due in large part to a perturbation of the NOS pathway. In most of these conditions, the abnormality is reversed or ameliorated by the administration of the NO precursor, L-arginine (Cooke and Dzau, 1997). L-arginine is metabolized by NOS to citrulline and NO.

Dr. John Cooke and coworkers were the first to demonstrate that endothelial vasodilator dysfunction could be reversed by administration of the NO precursor. In hypercholesterolemic rabbits, administration of L-arginine normalizes the NO-dependent vasodilation to acetylcholine (Girerd et al., 1990; Cooke et al., 1991b). Subsequently, Dr. Cooke and others have demonstrated that acute administration of L-arginine can reverse endothelial vasodilator dysfunction that is observed in the coronary and peripheral circulation in patients with atherosclerosis, and in subjects at risk for atherosclerosis.

Because NO has inhibitory effects on many of the key processes that promote atherosclerosis (monocyte adherence, platelet aggregation, vascular smooth muscle proliferation), Cooke postulated that chronic enhancement of vascular NO production could inhibit atherogenesis. Indeed, his lab demonstrated that in hypercholesterolemic rabbits, chronic oral administration of L-arginine could enhance vascular NO activity (Cooke et al., 1992; Wang et al., 1994; Tsao et al., 1994). This effect was associated

with a striking reduction in vascular lesions. By contrast, administration of NOS antagonists reduced vascular NO synthesis, increased endothelial adhesiveness for monocytes, and accelerated lesion formation (Tsao et al., 1994; Naruse et al, 1994; Cayatte et al, 1994). Cooke and others have shown that EDNO exerts its effects on atherogenesis by suppressing the expression and the signaling of endothelial adhesion molecules such as VCAM-1, and by reducing the expression of chemokines such as monocyte chemotactic protein-1 (Marui et al., 1993; Tsao et al., in press). The inhibition of adhesion signaling by NO appears to be mediated by cGMP, whereas the transcriptional effects of NO appear to be due, in part, to its abrogation of an oxidant-sensitive transcriptional pathway mediated by NFκB (Marui et al., 1993; Tsao et al., in press; Tsao et al., 1995).

Surprisingly, the administration of L-arginine in hypercholesterolemic rabbits with pre-existing lesions not only slows the progression of disease, but actually induces regression of atherosclerosis (Candipan et al., 1996).

Accordingly, enhancement of vascular NO may represent a novel therapeutic strategy for cardiovascular disease. The initial studies in humans are encouraging. Cooke and others have recently demonstrated that chronic oral administration of L-arginine in hypercholesterolemic humans or those with coronary artery disease can enhance vascular NO activity (as assessed by vascular reactivity studies and measurement of urinary nitrogen oxides), inhibit platelet aggregability, and reduce the adhesiveness of peripheral blood mononuclear cells (Bode-Böger et al., 1994; Wolfe et al., 1995; Theilmeier et al., in press; Lerman et al., 1997).

3. ADMA, a determinant of endothelial dysfunction and novel risk factor for atherosclerosis

ADMA (asymmetric dimethylarginine) is an endogenous antagonist of nitric oxide synthase. Several years ago, Vallance and Moncada demonstrated that, in uremic rats and in patients with renal failure, plasma ADMA levels were elevated 5-10-fold from normal values of about 1 micromolar (Vallance et al., 1992a,b). Plasma from uremic animals and patients (but not controls) induced the constriction of isolated

vascular rings. This vasoconstriction was reversed by L-arginine. Moreover, infusions of ADMA into the brachial artery of normal volunteers caused a significant increase in forearm vascular resistance at concentrations of ADMA that are found in patients with renal failure (Vallance et al., 1992b).

Recently, the enzyme that is responsible for degrading ADMA (dimethylarginine dimethylaminohydrolase, or DDAH), has been characterized. An antagonist to DDAH has been developed which blocks ADMA degradation (MacAllister et al., 1996). When the DDAH antagonist is added to vascular rings in vitro, a gradual increase in tone is observed. Again, this vasoconstriction is reversed by L-arginine. These studies suggest that ADMA is continuously being synthesized and degraded. An alteration in the turnover of ADMA can affect NO synthase activity.

Elevated levels of ADMA have been found in patients with hypercholesterolemia and atherosclerosis (Bode-Böger et al., 1996; Yu and Xiong, 1994).

ADMA is formed primarily by methylation of protein arginine inside cells, where it plays an important role in modulating protein-RNA interactions (Liu and Dreyfuss, 1995). Free ADMA is released upon protein turnover, and is probably secreted by most tissues and either passed in the urine or metabolized in the kidney (Tojo et al., 1997). Many types of physiological stress, such as the chronic inflammatory stress associated with atheroma formation, oxidative stress from environmental toxins, and stress which might result from poor nutrition, overweight, or age, is associated with chronic cellular damage and leads to increased rates of protein turnover, which in turn may lead to increased secretion of methylated amino acids and higher circulating levels of these amino acids, including ADMA. Indeed, excretory methylated amino acids have been widely used as markers of protein turnover in, for example, fasting or dystrophic animals (Mizobuchi et al., 1985; Bates et al., 1983).

Virtually all risk factors that are associated with accelerated atherosclerosis are also known to attenuate the synthesis and/or activity of EDNO. As a circulating antagonist of NO biosynthesis, ADMA may be an important determinant of endothelial vasodilator dysfunction, and potentially, an important new risk-factor for atherosclerosis. To further examine the role of ADMA and its importance in cardiovascular disease, methodology must be developed to detect ADMA with greater sensitivity, specificity, and with higher throughput.

## SUMMARY OF THE INVENTION

Cardiovascular disease assessment is improved by determining the value of the ADMA level in a subject's physiological fluid by itself, as an ADMA/L-arginine ratio or in conjunction with other know indicators of cardiovascular disease. Particularly, immunoassays are provided for the determination of the level of ADMA in the physiological fluid. Alternatively, the level of DDAH enzyme activity may be determined, by itself or in conjunction with ADMA, as an indication of the ability of the patient to hydrolyze ADMA and maintain an acceptable level of ADMA in plasma. Antibodies are provided which may be used in the immunoassays and effectively distinguish ADMA from close congeners. Kits may be provided which provide the various reagents for the ADMA assay and/or the DDAH assay, conveniently in conjunction with reagents for at least one of L-arginine, HDL, LDL, VLDL, Lp(a), triglycerides, homocysteine, chylomicron size distribution, or the like.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. is a graph of the plasma concentrations of ADMA as found in hypercholesterolemic and normo-cholesterolemic humans, as determined as described below;

Figure 2. is a graph of flow-mediated vasodilation of hypercholesterolemic subjects before and after intravenous infusion of the reagents;

Figure 3. is a bar graph of urinary nitrate excretion in hypercholesterolemic subjects before and after treatment as compared to normocholesterolemic control subjects; and

Figure 4. provides molecular structures of Arginine, ADMA and SDMA.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention ADMA is determined directly or vicariously, by determining the activity of DDAH. Conveniently, ADMA may be determined in any appropriate physiological fluid by an immunoassay, employing antibodies which provide for detection of ADMA with minimal interference from congeners, such as L-arginine and SDMA.

Methods for detecting ADMA in physiological fluids, such as blood, plasma and urine, include extraction, chemical derivatization, isolation by reverse-phase HPLC, GC-mass spectrometry, and fluorescence, although other methods may also be available. (Chen et al., 1997). The subject invention includes novel immunoassays employing antibodies which provide for sensitive detection of ADMA, without significant interference from congeners. These immunoassays may be modeled on immunoassays available today, where ADMA is substituted for the available analytes. The labels involved may be enzymes, fluorescers, chemiluminescers, particles, which particles may be opaque or clear, or may be conjugated with the previously indicated labels. The assay may involve spectrophotometric detection, fluorimetric detection, or visual detection, and may use any of a wide variety of reagents that are commercially available or may be developed along with methodologies described in the literature.

Determination of ADMA in clinical specimens is complicated by the presence of two closely related compounds which exhibit similar behavior and/or cross-reactivity in assays, namely arginine and symmetric dimethylarginine (SDMA). ADMA differs from arginine only in having two methyl groups on one of the guanidino nitrogens (see Figure 4). In normal human serum, the concentration of arginine is approximately 100 micromolar, or approx. 100 times that of ADMA. For

accurate clinical determination of ADMA, ADMA should comprise >90% of antibody-bound analyte. Thus, a useful antibody must have >1000-fold higher affinity (equilibrium association constant, Ka) for ADMA than for arginine, despite the small chemical difference.

SDMA is even more similar to ADMA than is arginine, having the two methyl groups on different guanidino nitrogens instead of on the same nitrogen (see Figure 4). In normal human serum, the concentration of SDMA is comparable to that of ADMA, or approx. 1 micromolar. Thus, by the above criterion a useful antibody must have >10-fold higher affinity for ADMA than for SDMA to insure that >90% of bound analyte is ADMA. As described in Example 1, we have isolated human Fab fragments which meet the required criteria in having affinities for ADMA of approx.$10^8$ $M^{-1}$, while having affinities for arginine and SDMA of $<10^5$ $M^{-1}$ and $<10^7$ $M^{-1}$, respectively.

The antibodies which are employed may be antisera from any convenient source, e.g. bovine, caprine, ovine, canine, equine, rodent, or the like, where the antisera may be purified by selecting out antibodies strongly binding to L-arginine and SDMA. The immunogen for production of the antibodies may be prepared by conjugating the ADMA at the alpha-amino group or the carboxyl group, particularly in the latter case using a linker for bonding to the antigen. Instead of antisera, monoclonal antibodies may be produced in accordance with known ways. Particularly, using mice, the mice may be immunized with the ADMA conjugate, splenocytes isolated and immortalized, and then screened for affinity for ADMA, against binding to L-arginine and SDMA. Clones of interest may be expanded and grown or their DNA isolated and the genes expressing the anti-ADMA heavy and light chains isolated and manipulated for expression in an appropriate host cell or host. Indeed, as described below, the genes may be mutated to further enhance binding affinity.

In referring to antisera, monoclonal antibodies and Fab fragments, it is understood that what is intended are binding proteins which have the general structure of the variable region of an antibody. These binding proteins will have a Ka of at least

about 5 x $10^6$, usually at least $10^7$, and preferably $10^8$, or higher. These binding proteins will comprise a region having substantially the structure and organization of the variable region ("Fv")of an antibody, where the heavy and light chain may be covalently or non-covalently joined. Other portions of an antibody may also be present, such as the first constant region.

The potential for using antibodies for therapy, recommended the production of antibodies having human Fc and conserved regions of the variable region. These would not be immunogenic and could therefore be administered repeatedly without loss of efficacy. Thus, we used a variable-region (V-region) repertoire library cloned from pooled non-immune human blood as the source (Hoogenboom et al., 1991; Marks et al., 1991a,b). A library of ~4x$10^{10}$ antibody Fab fragments displayed on filamentous bacteriophage was panned against immobilized ADMA to select ADMA-binding antibodies. To favor the selection of antibodies with high afinity for free ADMA but low affinity for arginine or SDMA, it was necessary to modify the standard panning strategy. First, soluble arginine and SDMA had to be present in the phage suspensions to inhibit antibodies with high affinities for these from binding to the immobilized ADMA. Secondly, two completely different structural forms of immobilized ADMA had to be used in tandem to favor selection of antibodies with high affinity for free ADMA. The presence of soluble arginine and SDMA favored selection of antibodies with high affinity for the composite epitopes of immobilized ADMA conjugates, but only weak affinity for free ADMA. Thus, selection of antibodies with high affinity for free ADMA could only be favored by tandem selection on two completely different structural forms of immobilized ADMA. In this way we were able to obtain human Fab fragments having affinities for free ADMA of $10^7$ - $10^8$ $M^{-1}$ and affinities for arginine and SDMA of $<10^4$ - $10^5$ $M^{-1}$ and $<10^6$ - $10^7$ $M^{-1}$, respectively. The affinities for ADMA as compared to arginine are at least$10^2$ greater, preferably at least $10^3$, and as compared to SDMA, are at least 10 fold greater.

Various protocols for immunoassays may be employed using a variety of reagents. One can provide for competition between a mimic of ADMA (a compound which competes with ADMA for binding to the binding protein, that is, has at least one common epitope) and any ADMA in the physiological specimen and measuring

the amount of the binding protein that binds to the ADMA mimic. This can be achieved by having the ADMA mimic bound to a solid surface, such as a wall of a vessel, e.g. a microtiter plate or a particle, which can be separated from the medium. One can use channeling where by bringing two of the binding proteins together, one obtains a different signal, e.g. fluorescence energy transfer. In this case one has a polyepitopic reagent which has at least two epitopes competitive with ADMA and two different binding proteins, one with a fluorescer which acts as a donor and one with a fluorescer which acts as a receiver. When the two different binding proteins are bound to the polyepitopic reagent there will be energy transfer, so that by using excitation light to excite the donor, one reads the emission wavelength of the receiver. A similar assay can be used with enzymes, where the product of one enzyme is the substrate of the other enzyme. One may have ADMA conjugated to a label, e.g. an enzyme, where binding of the protein to the conjugated ADMA changes the enzyme activity. Rather than labeling the binding protein, one can label a protein which binds to the binding protein. The anti-(binding protein) may be an antibody specific for an epitope of ADMA or the binding protein may be conjugated to a small molecule for which there is a strong binding protein, e.g. biotin and avidin, digoxin and antidigoxin, etc.

Enzyme-linked immunosorbant assays (ELISA) of one sort or another have been widely used for more than 25 years for the detection and measurement of analytes (primarily proteins) in bodily fluids (Harlow and Lane, 1988). Their principal advantages stem from the unparalleled affinities and specificities of monoclonal antibodies (mAbs) for analyte tagging, and the unparalleled catalytic power of enzymes for signal amplification. More recently the immunoassay repertoire has been expanded by the development of homogeneous solution phase assay formats which can be processed in fewer steps than ELISA with improved kinetics and sensitivity (Kopetzki et al., 1994; Coty et al., 1994; Henderson et al., 1986). The preferred format for the latter is based on readily assayable enzymes which have been modified in some way to make their activities sensitive to the presence of the target analyte. In the CEDIA system (cloned enzyme-dependent immunoassay), for example, the analyte is directly conjugated to the enzyme at a site which does not inhibit its activity except when the analyte adduct is bound by antibody (Coty et al., 1994; Henderson et al., 1986). When fluid samples containing free analyte are added to the system, the

inhibitory antibody is displaced from the enzyme, producing a proportional increase in activity which can be measured directly with chromogenic substrates.

In conventional ELISA haptenized ADMA is immobilized on the surface of microtiter plate wells (Harlow and Lane, 1988). Antibody is adsorbed out of solution by binding to the immobilized hapten, and after washing, the bound antibody is detected by binding a secondary antibody-enzyme conjugate which reacts with a chromogenic substrate, producing a signal which is propotional to the amount of bound anti-ADMA antibody (Harlow and Lane, 1988). Free ADMA in a clinical specimen is determined by diluting the specimen into the antibody solution prior to exposure of the antibody solution to the immobilized ADMA hapten. The resultng signal will be reduced by an amount proportional to the concentration of ADMA in the specimen, due to the fraction of antibody prevented from binding to the immobilized hapten by binding to free ADMA in the specimen. In practice, a series of two-fold dilutions of the specimen bracketing the expected concentration range is tested in triplicate and compared to standards of known ADMA concentration. The sensitivity of the assay is greatest when the antibody is used at a concentration no greater than ~0.1 x Kd. Under this condition, the specimen dilution which gives a 50% reduction of signal has an ADMA concentration equivalent to the Kd.

Fluorescence Polarization Immunassay (FPIA) is another homogeneous solution phase immunoassay for small molecule analytes, which has the unique advantage that a positive signal is generated by competition. FPIA detects the difference between antibody-bound and free fluorescently-labelled ligand as the polarization of emitted light when excited by plane-polarized light. Small molecule ligands tumble so fast during the excited state that emitted light is nearly isotropic, whereas, the ~500-fold larger antibody-bound ligand hardly rotates at all during the excited state and therefore emits highly polarized or anisotropic light. Analyte is measured by its ability to displace fluorescently-labelled analyte from analyte-specific antibody, or compete with label for binding to antibody, and thereby lower the polarization of emitted light, and increase its intensity at certain angles relative to the incident light. Sensitivity is a function of antibody affinity and the size difference between bound and free ligand. With nanomolar affinities and a ~500-fold size

difference between free and antibody-bound analyte, sub-picomolar concentrations may be detectable.

FPIA has the advantage that it is simple and homogeneous. FPIA is based on the principle that when a fluorescently-labeled molecule is excited with plane-polarized light, it emits light which is polarized to a degree which is proportional to the size of the molecule (Burke et al., 1996). This is because size is inversely proportional to rotation rate in solution and the farther an excited molecule rotates before emission, the less polarized the emitted light will be. Fluorescently-labeled small molecules rotate rapidly, thus, emitted light is less polarized, whereas, the same molecules rotate much more slowly when bound to an antibody, and therefore emitted light is much more polarized. ADMA may be conjugated to many fluorophores such as fluorescein by reaction of the free primary amino group of ADMA with an activated derivative of the fluorophore such as flurescein isothiocyanate (FITC). Free ADMA is measured as a function of its concentration-dependent ability to compete with fluorescein-ADMA for binding to antibody, thereby reducing polarized emission.

Additional immunoassay formats which could be used with our anti-ADMA antibodies include, but are not limited to, radioimmunoassay (RIA; Lauritzen et al., 1994), cloned enzyme donor immunoassay (CEDIA; Coty et al., 1994), biomolecular interaction analysis (BIA; Fägerstam et al., 1992), and fluorescence resonance energy transfer immunoassay (FRET; Youn et al., 1995). Like ELISA, most immunoassays for small, monovalent molecules are competitive inhibition assays in which the specimen analyte competes with labeled ligand for binding to antibody. In RIA the ligand is radioactive. In CEDIA the ligand is conjugated to the α-fragment of β-galactosidase, such that antibody-binding inhibits enzyme activity. Thus, competitive inhibition of the antibody-ligand interaction by specimen analyte results in an increase in chromogenic enzyme activity. In the FRET immunoassay the ligand is labeled with a fluorophore which can transfer its energy detectably to a fluorophore attached to the antibody only when both are in close proximity. BIA does not assay by competitive inhibition of binding, but rather directly monitors the interaction of specimen analyte with immobilized antibody using a phenomenon called surface

plasmon resonance, whereby the refractive index at the antibody-bound surface is detectably altered when analyte binds to the antibody.

If convenient, the assay may employ a fluorescence activated cell sorter and fluorescent particles employed. The assay would provide that the number of fluorescent particles counted would be related to the amount of ADMA present. For example, by having a competitive assay between particles to which ADMA is conjugated and ADMA in the specimen for fluorescently labeled binding proteins, the degree to which the particles are labeled with the fluorescent binding proteins will be proportional to the amount of ADMA in the specimen. One would then compare the number of fluorescent particles counted with the specimen as compared to a control value.

The following table organizes various assays which may find use in the subject invention.

| Immunoassay type | | Primary quantifier | Example |
|---|---|---|---|
| Competitive - *non-linear* proportionality of analyte to primary quantifier | Inverse | labeled antibody in complex with competitor | ELISA[1] |
| | | labeled competitor in complex with antibody | FPIA[2] |
| | Direct | ratio of unbound labeled competitor to bound | FRET[3] |
| | | unbound labeled competitor | CEDIA[4] |
| Non-competitive - *linear* proportionality of analyte to primary quantifier | | labeled antibody in complex with analyte | APEIA[5] |
| | | unlabeled antibody-analyte complex | BIA[6] |

1. Competitor is the immobilized antigen and bound label is inversely proportional to analyte.
2. Polarization of fluorescent label is inversely proportional to analyte.
3. Donor fluorescence is proportional to analyte, optional acceptor fluorescence is inversely proportional analyte.
4. Competitor is enzyme-analyte conjugate. Only free competitor is active and proportional to analyte.

5. Analyte protected enzyme immunoassay; only analyte-bound enzyme-antibody fusion is active. Available from Panorama Research, Inc., Mountain View, CA

6. Bimolecular interference analysis.

The physiological sample may be subject to prior treatment, depending on the nature of the sample and the nature of the assay. For whole blood, anticlotting factors may be included, alternatively, the red blood cells may be removed, the blood may be citrated or heparinized, etc. The sample may be concentrated or diluted, components precipitated out, the pH modified, particular buffers added, or the like. The untreated or treated sample may then be combined with the other reagents appropriate for the assay, incubated as appropriate and then assayed.

Where the ADMA concentration in plasma is greater than about 2μM, particularly greater than about 1.5μM, the subject may be considered to have a serious cardiovascular disease risk. The normal urinary excretion will generally be 13.5 ± 3.1 mg per 24 hours. The higher the level of ADMA, the more desirable it will be to determine other risk factors associated with cardiovascular disease, as described above. Of particular interest, will be the history of the subject, e.g. smoker, diabetic, etc., the total cholesterol, the HDL/LDL ratio, triglycerides, homocysteine and the like. In addition, monitoring of the subject will be warranted to watch for signs of cardiovascular disease and provide prophylactic treatment, such as providing excess L-arginine or L-lysine in the subject's diet, generally in addition to the normal diet, a supplement of from about 5 to 12 g/day. Other prophylactic regimens may also be employed, such as lower fat diets, increased fiber in the diet and the like.

The ADMA assay may be used as an initial assay, whereby a positive result, particularly a borderline result, in the range of 1 -2μM, more usually in the range of 1.5 - 2μM, would warrant further tests to corroborate the existence of the risk for cardiovascular disease.

While the determination of ADMA is found to be a superior predictor of cardiovascular disease risk, the other factors which are considered today aid in further enhancing the accuracy of the cardiovascular risk assessment. Normally, the greater the number of factors which are abnormal, the higher the risk for the subject. Other

common factors include L-arginine, HDL, LDL, VLDL, Lp(a), triglycerides, homocysteine, and chylomicron size distribution. The levels of these various factors present in a physiological fluid, where the factors may be determined individually or compared as ratios, may be used in conjunction with the ADMA determination in cardiovascular risk assessment. Kits may be provided where the reagents for the ADMA assay are made available in conjunction with the reagents for the determination of the other factors.

The ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) is a commonly-used ratio to assess risk of cardiovascular disease. However, the correlation of the arginine/ADMA ratio with key indicators of vascular dysfunction, namely NO-mediated vasodilatory response to blood flow ($R=0.631$, $p<0.01$), and urinary nitrate levels ($R=0.482$, $p<0.02$) was much better than that of total blood cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, age, or blood pressure (Böger, et al., submitted). Thus, the ratio of arginine to ADMA appears to be a much better predictor of endothelial vasodilatory dysfunction than is the LDL-to-HDL ratio. We believe that the arginine/ADMA ratio may be a more accurate predictor for cardiovascular disease than traditional risk factors because it is more reflective of the vascular response to hypercholesterolemia. The vascular response to hypercholesterolemia varies widely due to genetic background, but chronically elevated ADMA correlates much better with vascular dysfunction because it is a potentially causative precondition.

Disclosure of techniques used for determining various of the factors include: HDL, U.S. Patent Nos. 5,034,332; 5,451,370 and 5,460,974; LDL, U.S. Patent Nos. 4,126,416; 5,401,466 and 5,417,863; triglycerides, U.S. Patent Nos. 4,245,041 and 5,221,615; and homocysteine, U.S. Patent Nos. 4,940,658 and 5,631,127.

The kits which are provided will usually have the necessary reagents, depending upon the purchaser to have the equipment. Thus a kit will have at least labeled or unlabeled anti-ADMA, where in the latter case a labeled anti-(anti-ADMA) may be employed. Surfaces of vessels, microtiter plates, centrifuge tubes or the like, may be coated with the appropriate reagent (s) for detecting one or more of the factors.

Particles may be provided, which are coated with the appropriate reagents, e.g. antibodies, for isolating the analyte, analyte-receptor complexes, or the like. Reagents for developing a detectable signal, e.g. enzyme substrates may be included.

## EXPERIMENTAL

Examples:

1. Isolation of ADMA-specific mAbs which discriminate against arginine and SDMA.

As the source for anti-ADMA antibodies, we used a bacteriophage display library of Fab fragments cloned from peripheral blood lymphocytes of non-immune humans as described (Hoogenboom et al., 1991; Hoogenboom, 1997). Human antibody light-chain and heavy-chain variable (V) regions were amplified from peripheral blood lymphocyte RNA by reverse transcription and polymerase chain reaction (RT-PCR) using degenerate primers containing all the known 5' and 3' sequences of human V-regions. These libraries of V-region encoding fragements were then ligated into a phagemid vector for expression in the E. coli periplasm as Fab fragments fused to the amino terminus of the phage minor coat protein (gIIIp) via the Fd C-terminus. Fab fragments are comprised of complete light chain and heavy chain Fd, which is comprised of the V-region plus first constant region.

Between Fab and gIIIp in the expression product, three additional elements were encoded by the vector: (1) a 12-residue epitope tag (c-myc) for ELISA detection by anti-epitope antibody, (2) six-histidine tag for purification by affinity chromatography, and (3) a supressible stop codon (amber) for free Fab production in non-supressing hosts without the need for subcloning. The phage library was prepared by quantitative infection of E. coli strain TG1 cells (an amber-supressing host) expressing the Fab-gIIIp fusions with helper phage, which is essentially wildtype phage containing an antibiotic resistance gene. Phage particles which are produced by the infected cells contain Fab on their surface and the Fab-encoding phagemid inside. The library used for the present work was comprised of phage representing ~$4x10^{10}$ independent Fab clones.

Phage were panned against immobilized ADMA according to established procedures (McCafferty and Johnson, 1996; McCafferty, 1996). ADMA was conjugated through the alpha-amino group to epsilon-amino groups of exposed lysines on bovine serum albumin (BSA) via a suberate linker, and this conjugate was immobilized on a polystyrene surface. In the panning procedure a suspension containing ~$10^{13}$ phage particles was exposed to the immobilized ADMA conjugate for 1-2 hours to allow binding equilibration. The suspension contained BSA and suberate at 3% and 1 millimolar, respectively, to inhibit capture of Fab having high affinity for these components of the conjugate. Bound phage were washed and eluted with triethylamine. From the first round, $6.5x10^5$ phage were recovered. These were amplified in E. coli strain TG1 back up to ~$10^{13}$ and subjected to two more rounds of panning, i.e., binding, washing, elution, and amplification. In the second and third rounds, soluble arginine was included at 1 micromolar to prevent capture of Fab which cross-react with arginine. From the final round of panning $1.6x10^{10}$ phage were recovered. An aliquot of this phage population was used to infect E. coli strain HB2151. This strain does not suppress the amber stop codon between the Fd chain and gIIIp, and therefore the Fab is expressed in soluble form in the bacterial periplasm without the gIIIp domain.

Free Fab from 40 clones were screened by ELISA on the immobilized ADMA-suberate-BSA conjugate. 57% of these clones were found to give a positive ELISA signal with no detectable inhibition by soluble arginine, BSA, or suberate. However, none of the ELISA-positive clones showed inhibition by soluble ADMA at concentrations up to 1 micromolar. Thus, the presence of soluble components of the conjugate, including a structural analog of ADMA (arginine), biased the selection in favor of antibodies which had high affinities for the composite epitope of ADMA-suberate-BSA, but which had low affinities for each of the conjugate components alone, including ADMA. In order to identify rare clones which have high affinity for free ADMA, but low affinity for ADMA analogs such as arginine and SDMA, it was necessary to subject the third round phage eluate to additional rounds of panning against a completely different ADMA conjugate in the presence of the soluble ADMA analogs.

For the second conjugate, ADMA was linked, again through the alpha-amino group, to tosyl-activated magnetic beads (Dynabeads, Dynal Corp.). The phage population selected after three rounds of panning on ADMA-suberate-BSA were then subjected to four rounds of panning against ADMA-Dynabeads, without intervening amplification. This time the suspension contained 10 micromolar arginine and 100 nanomolar SDMA to inhibit capture of Fab with high affinities for these ADMA analogs. These concentrations were selected on the basis of the required level of discrimination for an antibody having a desired Kd ( $10^{-8}$ M). Approximately 2000 clones were recovered from this process. Soluble Fab from 600 of these clones were screened by competitive ELISA against immobilized ADMA-suberate-BSA in the presence and absence of 100 nanomolar soluble, free ADMA. Four clones were found to be inhibited by >50% by 100 nanomolar ADMA, and two of these clones, F and G, were inhibited by >80% by 100 nanomolar ADMA and by 20%-50% by 10 nanomolar ADMA. Thus, anti-ADMA Fab clones F and G have Kd in the $10^{-7}$-$10^{-8}$ M range. Both clones showed no detectable inhibition by up to 10 micromolar arginine and by up to 1 micromolar SDMA. Thus, anti-ADMA Fab clones F and G are judged to have the necessary affinities and specificities to allow accurate determination of ADMA in clinical specimens without detectable interference by arginine or SDMA.

For further characterization and use in clinical assays, Fab were purified from the supernatants of large-scale bacterial cultures of clones F and G. Ultrafiltration was used to increase the concentration approximately 40-fold and replace the bacterial growth medium with phosphate-buffered saline (PBS). Fab were then affinity-purified by the six-histidine tag at the carboxy terminus of each encoded by the expression vector, using immobilized metal ion affinity chromatography (IMAC; Janknecht et al, 1991). Fab yields were typically 0.5-1 mg per liter and were judged to be >90% pure by silver-stained SDS-PAGE.

The purified Fab were retested for ELISA performance and conditions were optimized. When microtiter wells were coated with 1 microgram ADMA-suberate-BSA, $1x10^{-9}$ M Fab gave an adequate signal (1-2 OD405 in 30' with 0.1-0.2 OD background) with anti-myc-tag mouse antibody, horseradish peroxidase-conjugated (HRP) rabbit anti-mouse antibody, and ABTS, a chromogenic

substrate for HRP. Under these conditions, i.e., when the antibody concentration is ($\leq$0.1 x Kd), the concentration of free ADMA at which the background-corrected signal is reduced by 50% is equal to the Kd. When a range of ADMA concentrations from $10^{-5}$ M to $10^{-10}$ M was assayed with $10^{-9}$ M Fab, 50% inhibition was observed to occur between $10^{-7}$ M and $10^{-8}$ M, consistent with the original estimates of Kd. No detectable inhibition was observed with 100-fold higher concentrations of arginine or equivalent concentrations of SDMA. Competition ELISA is most sensitive when the analyte concentraion is equivalent to the Kd, i.e. at 50% inhibition, and such ADMA concentrations correspond to a 10 - 100-fold dilution of healthy serum. Thus, the Fab clones E and F have sufficient affinity and specificity for accurate clinical determination of ADMA by competition ELISA.

An additional confirmation of the Kd was obtainied by Scatchard analysis of the absorbance data. When the ratio of the concentration of the Fab-ADMA complex to the concentration of free ADMA is plotted against the concentration of the complex, Kd may be obtained from the slope (-1/Kd). The complex concentration is equal to the total Fab concentration times $(A - A_b)/(A_f - A_b)$ , where $A_f$ is the A405 of the Fab in the absence of ADMA and $A_b$ is the A405 of the Fab in an excess of ADMA, i.e. a concentration of >100 x Kd, e.g., $10^{-5}$ M. Free ADMA is equal to the total ADMA minus the complex.

2. Determination of serum ADMA using anti-ADMA Fab clones F and G in competition ELISA.

Blood samples from healthy human subjects are employed After removal of cells from the serum, ADMA is determined by the standard method, which involves removal of serum proteins, fluorescent labeling with o-phthalaldehyde, reversed phase high performance liquid chromatography (HPLC), and post-column, in-line fluorometric detection (Chen et al., 1997). The same specimens are assayed by competition ELISA essentially as described in Example 1 with the Fab concentration at $1x10^{-9}$ M. A series of dilutions of each specimen ranging from 1:10 to 1:100 is assayed in triplicate, and the results are compared to those of the standard method and

a standard curve of pure ADMA from $10^{-9}$ M to $10^{-7}$ M. One observes excellent agreement between the standard assay and competition ELISA.

3. Optimization of FPIA using anti-ADMA Fab clones F and G.

ADMA was conjugated through its alpha-amino group to Oregon Green (OG) and purified by reversed phase HPLC. The fluorescence polarization of the free conjugate was determined with a polarizing fluorometer at 498nm excitation maximum and 524nm emission maximum over a range of concentrations from $10^{-10}$ M to $10^{-8}$ M. The polarization of ADMA-OG was found to be fairly constant over the entire range with a value of 20 ±5 at $10^{-9}$ M being typical. At $10^{-10}$ M ADMA-OG, polarization was determined after equilibration with various concentrations of Fab ranging from $10^{-10}$ M to $10^{-6}$ M. As expected the minimum value was equivalent to that of the free conjugate and was reached between $10^{-9}$ M and $10^{-10}$ M. The maximum value of (150 - 200) was reached between $10^{-7}$ M and $10^{-6}$ M. The inflection point occurred between $10^{-7}$ M and $10^{-8}$ M, consistent with previous estimates of the Kd, and this was confirmed by Scatchard analysis of the fluorescence polarization data. In this case, the ratio of Fab-ADMA-OG complex to free Fab is plotted against the complex and, again, the Kd is derived from the slope (-1/Kd). The complex concentration is equal to the total ADMA-OG concentration times $(P - P_f)/(P_b - P_f)$, where $P_f$ is the polarization of free ADMA-OG and $P_b$ is the polarization of ADMA-OG in the presence of an excess of Fab, i.e., >100 x Kd, e.g., $10^{-5}$ M. Free Fab is equal to total Fab minus the complex. When the Fab is at 5 x $10^{-8}$ M (≅Kd) and ADMA-OG is at $10^{-9}$ M, polarization is 50% of maximum. This is the region of maximum sensititvity of the assay. When polarization is measured in the presence of $10^{-7}$ M free ADMA, which corresponds to a 10-fold dilution of healthy serum, polarization is 9% of maximum, representing an 82% inhibition. No detectable inhibition was observed with 100-fold higher concentration of arginine, or with an equivalent concentration of SDMA. Thus, the Fab from clones E and F have sufficient affinity and specificity for accurate clinical determination of ADMA by FPIA.

4. Determination of serum ADMA using anti-ADMA Fab clones E and F in FPIA.

As discussed above, optimum sensitivity of fluorescence polarization to competitive inhibition occurs when the antibody concentration is equivalent to Kd and the fluorescent ligand concentration is (0.1xKd). Under these conditions, uninhibited polarization is at the mid-point of its range, and ~80% inhibition can be achieved with a 100-fold excess of the free analyte. The same specimens analyzed in Example 2 are analyzed again by FPIA with the Fab concentration at 1xKd, and the ADMA-OG concentration at 0.1x Kd. A range of dilutions from 1:2 to 1:100 are assayed and compared to a standard curve ranging from $10^{-6}$ M to $10^{-8}$ M. Again, the results are observed to agree with the standard assay as well as with the competition ELISA.

It is evident from the above results that the subject invention provides for an early warning risk factor for cardiovascular disease. By incorporating a determination of ADMA in the assessment of the health of a subject, an early indication of risk of cardiovascular disease is given. This determination may be further buttressed with other symptoms and other assays, as well as lifestyle and the like. The subject invention provides for highly sensitive assays which have low interference from congeners, so as to have low false positives and negatives. Binding proteins are provided which allow for successful assays for ADMA without significant interference from L-arginine and SDMA.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A binding protein comprising an Fv and having an affinity for asymmetric N,N-dimethyl arginine ("ADMA")of at least 1 x $10^7$ and at least about a 10-fold less binding affinity for symmetric N,N'-dimethyl arginine and at least about a $10^3$ -fold less binding affinity for arginine.

2. A binding protein according to Claim 1 comprising a human Fv.

3. A binding protein according to Claim 1 comprising at least the first constant region of an antibody.

4. A binding protein according to Claim 1 comprising a detectable label.

5. A binding protein according to Claim 4, wherein said detectable label is a light absorber, fluorescer, chemiluminescer, enzyme, or radioisotope.

6. In a method for assessing risk of a subject for cardiovascular disease, the improvement which comprises:

determining the level of ADMA in a physiological fluid of said subject as compared to the level of a normal subject, where an elevated level indicates an enhanced risk for said cardiovascular disease.

7. A method according to Claim 6, wherein said determining comprises the use of a binding protein according to Claim 1.

8. A method according to Claim 7, where said determining comprises the use of a fluorescent immunoassay.

9. A method according to Claim 7, where said determining comprises the use of an enzyme immunoassay.

10. A method for determining risk of cardiovascular disease, said method comprising:

combining a blood specimen from a human subject with a binding protein comprising an Fv and having an affinity for asymmetric N,N-dimethyl arginine ("ADMA")of at least $10^7$, at least about a 10 fold less binding affinity for symmetric N,N'-dimethyl arginine and at least about a $10^3$-fold less binding affinity for arginine, wherein said binding protein is bound to a detectable label selected from the group consisting of fluorescers and enzymes; and

determining the amount of binding protein bound to said ADMA, wherein an amount of ADMA above normal indicates an enhanced risk for cardiovascular disease.

11. A method according to Claim 10, wherein a molecule competitive with ADMA for binding to said binding protein is bound to a solid surface and said determining comprises detecting the amount of label bound to said surface.

12. A method according to Claim 10, wherein said label is bound to said binding protein with a labeled anti-(binding protein).

13. A kit comprising a binding protein according to Claim 1 and any additional reagents neccessary for determining the amount of ADMA in a physiological specimen.

14. A kit according to Claim 13, further comprising reagents for determining at least one of L-arginine, Lp(a), HDL, LDL, triglycerides and homocysteine.

15. A kit according to Claim 14, wherein said binding protein is bound to a detectable label which is a member of the group consisting of fluorescers and enzymes or said kit comprises an anti-(binding protein) to which is bound a fluorescer or enzyme.

16. A kit according to Claim 13, wherein said binding protein is bound to a detectable label which is a member of the group consisting of fluorescers and enzymes or said kit comprises an anti-(binding protein) to which is bound a fluorescer or enzyme.

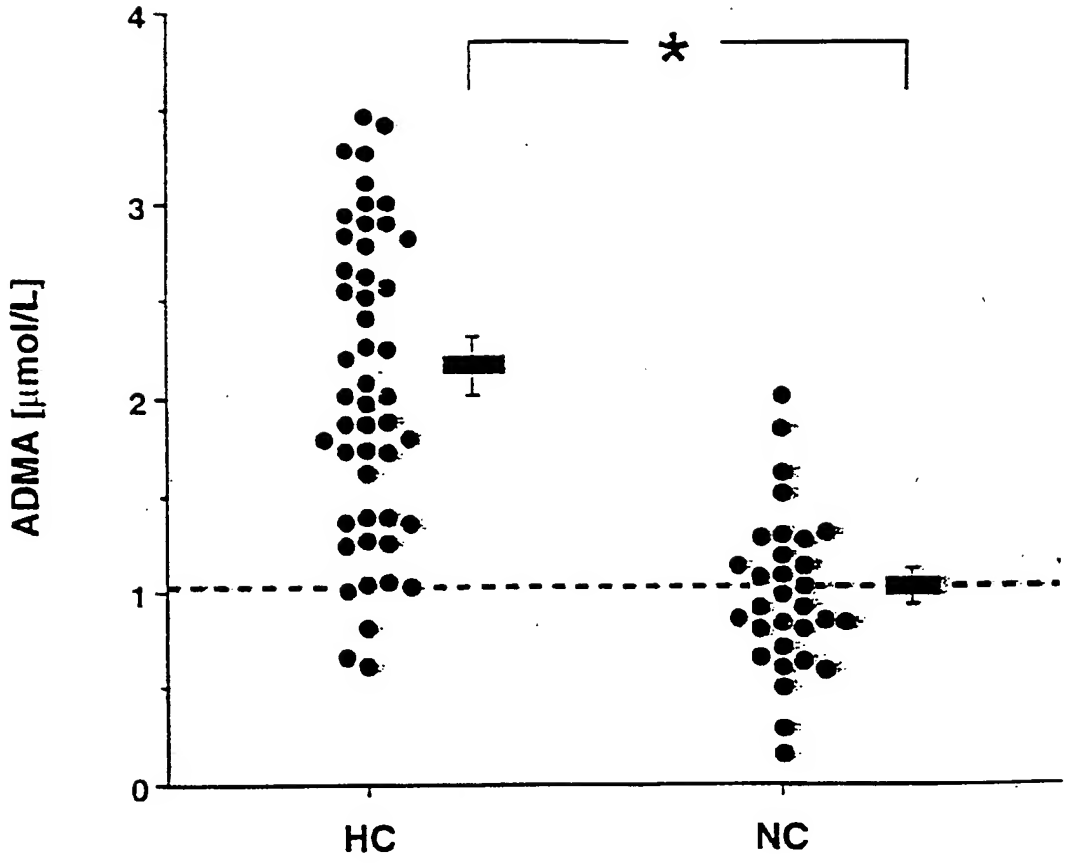


Figure 1

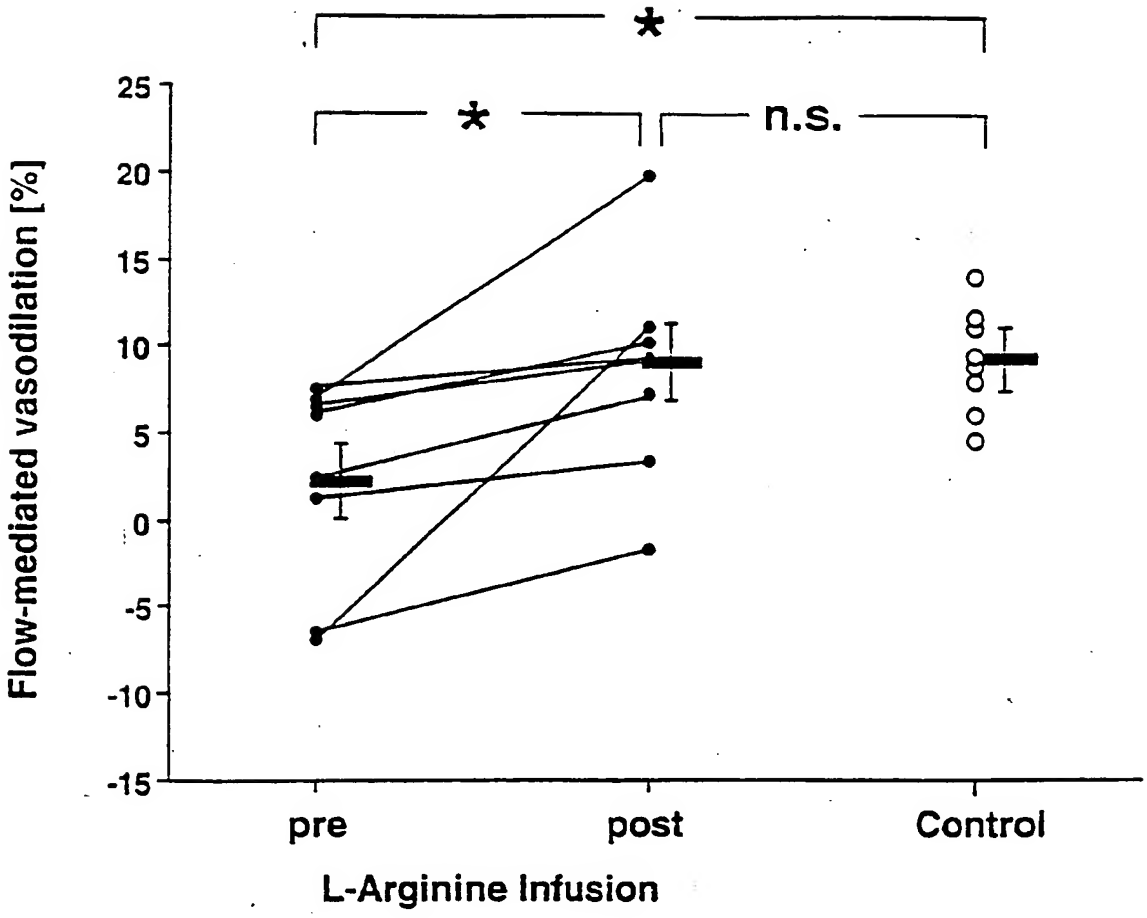
*
*
n.s.
25
20
15
10
5
0
-5
-10
-15
Flow-mediated vasodilation [%]
pre
post
Control
L-Arginine Infusion

Figure 2

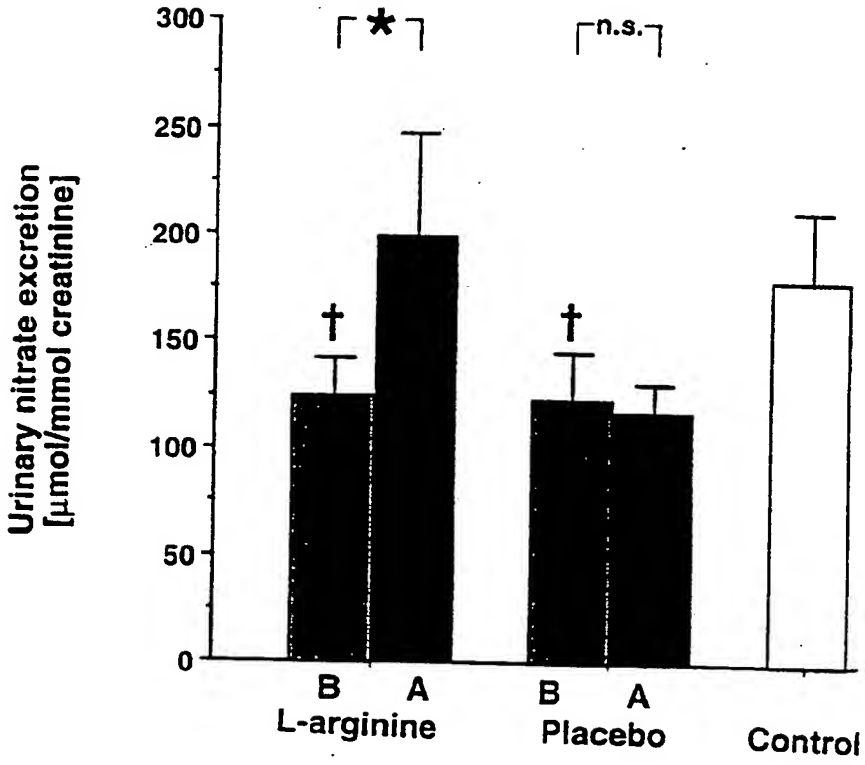
Urinary nitrate excretion
[µmol/mmol creatinine]
300
250
200
150
100
50
0
*
n.s.
†
†
B
A
B
A
L-arginine
Placebo
Control

Figure 3

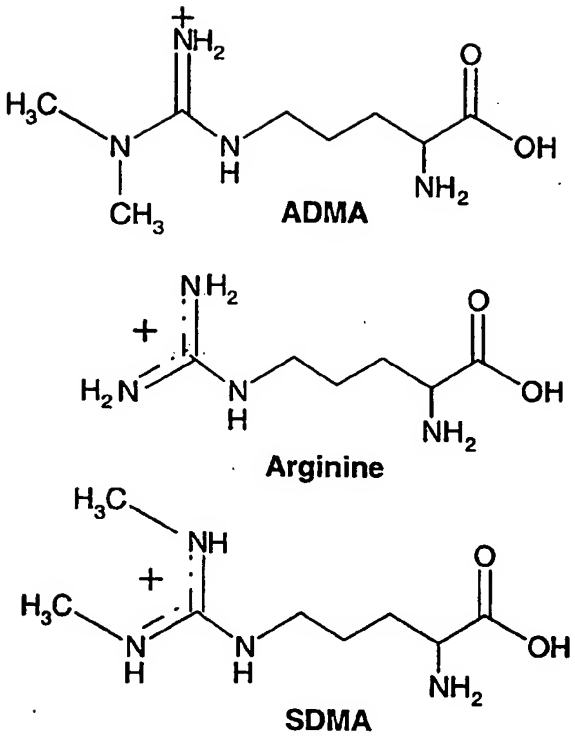


**Figure 4**

# INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/08720

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/00; C12Q 1/60; G01N 33/00, 33/53, 33/92

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | JIN, J.S. et al. Central and Peripheral Effects of Asymmetric Dimethylarginine, an Endogenous Nitric Oxide Synthetase Inhibitor. Journal of Cardiovascular Pharmacology. 1996. Vol. 28, No. 3, pages 439-446, see entire document. | 6-16 |
| Y | VALLANCE, P. et al. Accumulation of an endogeneous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 07 March 1992. Vol. 339, pages 572-575, see entire document. | 6-16 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 JULY 1998

Date of mailing of the international search report

04 SEP 1998

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INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/08720

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Dialog SciSearch Abstract No. 03768651 posted 1995. Ritz, E. et al. ADMA and Mortality in Dialysis-Role of Asymmetric Dimethyl-L-Arginine. Nieren-und Hochdruckkrankheiten. December 1994. Vol. 23, No. S2, pages S116-S120., see entire document. | 6-16 |
| Y | US 4,126,416 A (SEARS) 21 November 1978, see entire document. | 14 |
| Y | US 4,245,041 A (DENNEY) 13 January 1981, see entire document. | 14 |
| Y | US 4,940,658 A (ALLEN et al.) 10 July 1990, see entire document. | 14 |
| Y | US 5,034,332 A (RAPACZ et al.) 23 July 1991, see entire document. | 14 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08720

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 7.9, 11; 436/71, 86, 548; 530/387.1, 388.9, 389.1, 389.8

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/7.1, 7.9, 11, 968, 973, 975; 436/71, 86, 548, 804, 808, 811, 822; 530/387.1, 388.9, 389.1, 389.8

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG
search terms: dimethyl arginine, adma, sdma, antibody, binder, receptor, ligand, cardiac, vascular, cardiovascular